GIBSON DECLARATION-EXHIBIT 4

Vaccine 36 (2018) 5460–5469

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Will we have new pertussis vaccines?

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ARTICLE INFO

Article history:
Available online 24 November 2017

Keywords:
Pertussis
Acellular vaccines
Whole-cell vaccines
Live attenuated vaccines

ABSTRACT

Despite wide vaccination coverage with efficacious vaccines, pertussis is still not under control in any country. Two types of vaccines are available for the primary vaccination series, diphtheria/tetanus/whole-cell pertussis and diphtheria/tetanus/acellular pertussis vaccines, in addition to reduced antigen content vaccines recommended for booster vaccination. Using these vaccines, several strategies are being explored to counter the current pertussis problems, including repeated vaccination, cocoon vaccination and maternal immunization. With the exception of the latter, none have proven their effectiveness, and even maternal vaccination is not expected to ultimately control pertussis. Therefore, new pertussis vaccines are needed, and several candidates are in early pre-clinical development. They include whole-cell vaccines with low endotoxin content, outer membrane vesicles, new formulations, acellular vaccines with new adjuvants or additional antigens and live attenuated vaccines. The most advanced is the live attenuated nasal vaccine BPZE1. It provides strong protection in mice and non-human primates, is safe, even in immune compromised animals, and genetically stable after *in vitro* and *in vivo* passages. It also has interesting immunoregulatory properties without being immunosuppressive. It has successfully completed a first-in-man clinical trial, where it was found to be safe, able to transiently colonize the human respiratory tract and to induce immune responses in the colonized subjects. It is now undergoing further clinical development. As it is designed to reduce carriage and transmission of *Bordetella pertussis*, it may hopefully contribute to the ultimate control of pertussis.

1. Introduction

In spite of a roughly 85% global vaccination coverage with up to 99% coverage in many countries, pertussis, also referred to as whooping cough, has not yet been controlled anywhere. In fact, the disease has made a spectacular resurgence in several industrialized countries with very high vaccination coverage. Pertussis is a severe, infectious and highly contagious respiratory disease. It affects all age groups and can be fatal, especially in very young infants.

2. Current pertussis vaccines

2.1. Whole-cell pertussis vaccines

Soon after the discovery of its main causative agent, *Bordetella pertussis*, first successful attempts to limit disease severity and to control an epidemic by vaccination with a whole-cell extract of *B. pertussis* have been reported [1]. Whole-cell vaccines, consisting of heat- or formalin-inactivated *B. pertussis* bacteria and combined with diphtheria- and tetanus-toxoid-based vaccines as DTP vaccines have been shown by pioneering clinical efficacy trials to be highly efficacious after three doses, especially against severe pertussis [2]. Since the massive implementation of DTP vaccines in the 1950s and 1960s, the disease burden has markedly lessened [3]. However, doubts about the safety of these vaccines, starting to be voiced already in the early 1960s, have led to the suspension or rejection of DTP vaccines in several countries, such as Sweden, Japan and the United Kingdom. These adverse reactions ranged from local swelling and pain, to systemic reactions, such as fiver, irritability, excessive crying and, in rare cases to encephalopathy [4]. The almost immediate recurrence of the disease after cessation of vaccination [5] strongly illustrates the effectiveness of the DTP vaccines to limit pertussis disease burden.

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https://doi.org/10.1016/j.vaccine.2017.11.055
0264-410X/

2.2. Acellular pertussis vaccines

Nevertheless, distrust in the DTP vaccines has led to a general decrease in vaccination coverage in several countries, but also to efforts to develop safer and less reactogenic pertussis vaccines. These second-generation, acellular pertussis vaccines are composed of purified *B. pertussis* antigens, also combined with diphtheria and tetanus toxoids and are referred to as DTaP. They vary in antigenic composition from one to five *B. pertussis* antigens. Developed in Japan in the late 1970s, and then used in the early 1980s, the first acellular vaccine, consisting of purified pertussis toxin (PT) and filamentous haemagglutinin (FHA) [6], was subsequently evaluated for its reactogenicity and protective efficacy and compared to a vaccine containing only PT in a placebo-controlled phase III trial in Sweden [7]. The acellular vaccines were found to be less reactogenic than whole-cell vaccines [6] and highly effective in preventing pertussis disease [6,7]. Depending on the case definition, protection ranged from 50% to 80% and was better against severe disease than against the milder forms. These studies were followed by direct head-to-head comparative trials with different acellular vaccines, which included, in addition to PT and FHA, pertactin and two fimbrial serotypes, and with a whole-cell vaccine. A study enrolling close to 10,000 infants confirmed the improved safety profile of the acellular vaccines, compared to the whole cell vaccine [8]. It also showed that all the acellular vaccines were efficacious against typical pertussis disease, but that the five-component vaccine was more efficacious against mild disease than the mono-component or two-component vaccines. Curiously, in this study the whole-cell vaccine provided a protective efficacy of less than 50%, whereas efficacy reached 85% for the five-component acellular vaccine. A study simultaneously performed in Italy came to the same conclusion that the tested acellular vaccines, composed of PT, FHA and pertactin, were more efficacious that the whole-cell comparator [9]. However, in both studies, the protection conferred by the whole-cell vaccine, which was identical in both studies, was surprisingly low. In another trial, conducted in Senegal, in which a different whole-cell vaccine was compared to a two-component acellular vaccine, did not confirm superior efficacy of the acellular vaccine over the whole-cell vaccine, although it confirmed improved safety. In fact, DTaP recipients showed a significantly greater rate of pertussis than DTwP recipients [10]. In addition, this study revealed a shorter duration of protection procured by the acellular vaccine, compared to the whole-cell vaccine.

These apparently conflicting observations illustrate the difficulties to manufacture consistently potent whole-cell vaccines. On the other hand, the efficacies of the acellular vaccines produced by different manufacturers tested in these trials were comparable when the same case definitions were used, suggesting a more robust consistency in the preparation of acellular vaccines. Together with an improved safety profile of the acellular vaccines, these considerations led many industrialized countries to gradually switch from whole-cell to acellular vaccines in the late 1990s, early 2000s. In most European countries, the US, Canada, Australia and Japan acellular vaccines are now exclusively used. The improved safety profile of acellular vaccines has increased the vaccine acceptance rate, which, as a consequence, has led to a second decline in pertussis incidences in countries where vaccination coverage had increased again [6,11]. Nevertheless, most countries in the world continue to use whole-cell vaccines, mainly due to cost issues and limited production capacities.

3. Current pertussis problems

Since the implementation of the acellular pertussis vaccines in most of the industrialized countries, a sudden and unexpected upswing in pertussis incidence and associated death rates has been observed, despite continuously high vaccination coverage [12–15]. However, a trend of this resurgence was already observed before the introduction of acellular vaccines, and some countries in which whole-cell vaccines are still in use also experience a rebound of pertussis [16]. Nevertheless, the introduction of acellular vaccines seems to have accelerated this resurgence. In Queensland, Australia, DTaP has replaced DTP in 1999 for the primary course of immunizations, implying that in the beginning the children received a primary course of only DTP, only DTaP or a mix of both. Ten to twelve years later, the pertussis incidence increase could be linked to the nature of the vaccines used for the primary vaccination. Higher rates of pertussis were found in children who had received 3 DTaP administrations compared to those who had received 3 DTP doses [17]. Intermediate pertussis rates were found in children who had received a mix, provided that the first dose was a DTP. Thus, priming with DTP was associated with a lower risk of developing pertussis than priming with DTaP, a difference that persisted for more than a decade. Subsequent studies have confirmed the unexpected rapid waning of effectiveness of acellular pertussis vaccines in other countries [18].

Rapid waning of acellular pertussis vaccine-induced immunity may thus be one of the reasons for the current resurgence in countries with high vaccination coverage. However, other reasons may also contribute [19]. They include increased awareness and better diagnostic tests, sub-optimal use of current vaccines, antigenic mismatch between the vaccines and circulating strains and lack of protection against *B. pertussis* transmission.

Numerous studies have shown allelic variations in *B. pertussis* that have evolved over the years since the introduction of mass vaccination [20]. However, whether this is a major driver in the current resurgence of pertussis, is still a matter of debate. Nevertheless, it is striking that since the introduction of acellular vaccines in many countries an increasing proportion of recent clinical isolates lack pertactin, one of the major antigens included in most acellular vaccines. This has not occurred in countries in which whole-cell vaccines are still in use [21]. In the US, 85% of the clinical isolates collected between May 2011 and February 2013 from 8 states did not produce pertactin [22]. Different molecular mechanisms were identified that account for pertactin deficiency. They ranged from point mutations in the pertactin coding region or its promoter to insertions and deletions, indicating that pertactin-deficient strains did not emerge by clonal expansion and argues for a selective advantage of strains lacking pertactin. In addition, the odds of infection by pertactin-deficient *B. pertussis* were higher in fully vaccinated compared to unvaccinated subjects, suggesting that this selective advantage was vaccine-driven. Importantly, the clinical manifestations of whooping cough caused by pertactin-negative or pertactin-positive *B. pertussis* are virtually indistinguishable [23], indicating that pertactin deficiency does not alter the disease severity. Studies in mice have suggested that pertactin-deficient strains have a competitive advantage over pertactin-producing strains to colonize the mouse respiratory tract in the context of vaccination with acellular pertussis vaccines, whereas in the absence of vaccination, this competitive advantage was not apparent [24].

Whereas the expansion of vaccine escape mutants and rapid waning of acellular vaccine-induced immunity have a likely contribution to the resurgence of pertussis, a third reason may be the inability of the current vaccines to prevent infection by and transmission of *B. pertussis*. For a long time, transmission of *B. pertussis* has been difficult to study in non-clinical models. The recent development of a baboon model offers now the possibility to study this in non-human primates. Acellular pertussis vaccines have been shown to protect against pertussis disease in baboons, but fail to prevent infection in this model [25]. Three human DTaP or DTP

doses given at 2, 4 and 6 months of age were equally effective in preventing cough and leucocytosis upon infection with a highly virulent clinical isolate of *B. pertussis*. However, they did not protect against infection, although DTP-vaccinated animals cleared the infection faster than DTaP-vaccinated baboons. In fact, DTaP-vaccinated baboons carried the infection longer than the non-vaccinated animals. In addition, DTaP-vaccinated baboons could readily be infected by natural transmission and were able to transmit *B. pertussis* to naïve contacts. In contrast to vaccinated baboons, convalescent baboons were fully protected against infection by a second *B. pertussis* challenge. If this baboon model truthfully reflects the human situation, these observations suggest that vaccination neither prevents infection nor circulation of the organism.

That vaccination does not prevent *B. pertussis* infection in humans, nor the circulation of the organism in human populations in any important manner, comes from the observation that the inter-epidemic intervals have not changed in a major way since the implementation of mass vaccination [26]. A vaccine that prevents infection is expected to increase the inter-epidemic time intervals or to abolish the regular cycles altogether, since it would induce herd immunity, as observed for measles vaccines. Pertussis occurs in 3–5 years cycles, and mass vaccination, even with DTP, has only increased the inter-epidemic intervals by 1–2 years [27]. Thus, even DTP has had only a minimal herd immunity effect, not sufficient to interrupt the circulation of the organism in the human population. In fact, a recent mathematical modelling study concluded that asymptomatic transmission is the most parsimonious explanation for the resurgence of pertussis in countries with high-vaccination coverage [28], stressing the importance of herd immunity for ultimate control of pertussis, and hence the need for a vaccine that not only protects against pertussis disease, but also against infection by *B. pertussis*.

4. Attempted solutions to the current pertussis problem

4.1. Booster vaccinations

Given the relatively fast waning of acellular pertussis vaccine-induced immunity, repeated vaccinations have been recommended. However, the reactogenicity of acellular pertussis vaccines increases with increasing numbers of doses. Therefore, vaccine formulations with reduced antigen contents have been developed for the administration as booster vaccines for older children, adolescents and adults. Reducing the antigen content of combined acellular pertussis vaccines, referred to as Tdap vaccines, did apparently not affect antibody production against the *B. pertussis* antigens, when given as a booster vaccine in older children [29], but was better tolerated and resulted in fewer local reactions than DTaP [30]. The use of Tdap as a booster dose for adolescents aged 11–12 years was then recommended by the US Advisory Committee on Immunization Practices (ACIP) [31] and later for booster vaccination between 11 and 64 years of age. Since 2012 ACIP also recommends Tdap vaccination for all persons aged 65 years or older [32]. However, even with Tdap, the large injection site reactions were not totally eliminated [33]. Furthermore, a recent case-centered study investigating acute demyelinating events following vaccination based on several millions of vaccine doses given detected a slight, but statistically significant increased risk of acute disseminated encephalomyelitis 5–28 days following Tdap vaccination, although the number of cases was extremely low [34]. Serum antibody levels to *B. pertussis* antigens were found to be strongly induced by Tdap boosters, but decline rapidly, within one year after vaccination in adolescents and adults [35]. Several effectiveness studies have been carried out and showed that Tdap vaccination was only moderately effective in 11–19 years old students during an outbreak in Maine from 2010 to 2011 [36]. Especially in adolescents who had only received DTaP during the primary series of immunization, Tdap boosting provided only moderate (less than 70%) effectiveness, which waned very rapidly to reach less than 10% by ≥4 years after vaccination [37].

4.2. Cocoon vaccination

Obviously the main goal of pertussis immunization programs is the protection of young children against severe disease and pertussis-linked mortality. One of the strategies proposed to achieve this goal is the "cocoon strategy", consisting of vaccinating parents, siblings and other household members of a new-born child [38]. Cocoon vaccination has been recommended in several countries, including France [39]. It is based on the fact that parents, and especially mothers, are the primary source of *B. pertussis* infection of new-borns [40], and on the notion that vaccination of the household members should induce herd immunity and thereby prevent transmission of the organism to the neonates. There is no evidence, however, that DTaP or Tdap vaccination prevents *B. pertussis* infection and subsequent transmission [25]. Furthermore, cocoon vaccination is difficult to implement at levels needed to be effective [39,41], as estimated in mathematical modelling studies [42]. However, again these studies are based on the assumption that current vaccines are able to prevent infection and transmission. Effectiveness studies on cohorts in which cocoon vaccination was successfully implemented showed that postpartum vaccination of mothers did not reduce the number of pertussis cases in their children [43]. It was felt therefore that post-partum vaccination of mothers was not sufficient to have an impact on the occurrence of infant pertussis, and that other household members should be included. A subsequent study then evaluated the cocoon effectiveness through a program in which free Tdap vaccination was made available to all household contacts of new-born infants [44]. This study also failed to show a beneficial effect of a cocooning program on infant pertussis. On the other hand, a case-control study conducted in Australia found cocoon vaccination to decrease he risk of pertussis in infants by approximately 50% [45]. However, a more recent population-based study, also conducted in Australia, found that cocoon vaccination did not reduce pertussis diagnosis in children [46]. Thus even complete cocooning has at best a marginal effect on the prevention of infant pertussis.

4.3. Maternal vaccination

A more promising strategy to reduce infant pertussis appears to be maternal immunization. This strategy is based on the fact that maternal immunoglobulins are actively transported through the placenta to the foetus after 30 weeks of gestation [47]. Through this mechanism it is then expected that Tdap vaccination after 30 weeks of gestation will result in sufficiently high antibody levels in the foetus so that the new-born will be protected during the first week after delivery, until immunity is taken over by the primary vaccination of the infants. Placental transfer of maternal anti-*B. pertussis* antibodies to the foetus after vaccination during the third trimester of pregnancy has indeed been well documented [48]. These antibodies could be detected in the infants up to 2 months after birth. However, in contrast to anti-diphtheria and anti-tetanus antibodies, it is not known how anti-*B. pertussis* antibody levels translate into clinical benefit for protection against the disease. Efficacy or effectiveness studies are therefore required to assess the benefit of maternal Tdap vaccination. One of the first effectiveness studies on maternal Tdap vaccination reported en estimated effectiveness of over 90% against pertussis disease in the offspring [49]. High effectiveness of maternal immunization was rapidly confirmed in subsequent studies [50], and Tdap

vaccination at each pregnancy between 28 and 38 weeks of gestation is now recommended in several countries, including the US, the United Kingdom, Argentina, Belgium, Israel and New Zealand. Although, these studies are very encouraging, maternal immunization appears difficult to implement [51], albeit conceivably easier and more cost-effective than cocoon vaccination with Tdap. Furthermore, the effect of repeated vaccination at each pregnancy and the effect of maternal vaccination on the effectiveness of the primary infant vaccination are not yet fully understood. Previous studies have shown an inverse relationship between the presence of maternal antibodies and DTP-induced antibodies in the infants [52]. This seems to be less the case if DTaP is used for the primary vaccination, although more recent reports have also shown a certain level of blunting of the DTaP-induced antibody levels by maternal antibodies [53]. Considering that in most countries DTP is still used for the primary vaccination, these observations have to be taken into account, although the clinical relevance of this blunting effect still needs to be established. Studies in mice have shown reciprocal interference of maternal and infant immunization in the protection against pertussis challenge [54]. Whether this might be also the case in humans remains to be seen.

Although maternal vaccination may be a good solution to the neonatal pertussis problem in the short run, it is unlikely to interrupt transmission of the causative agent and therefore will probably not result in ultimate control of pertussis. If the question asked were "Do we need new pertussis vaccines?", the answer would thus be definitely yes. New vaccines are needed that not only provide long-lasting protection against the disease but also against infection and thereby prevent transmission.

5. New vaccines against pertussis

Since the implementation of DTaP and Tdap very few new pertussis vaccines have been in clinical development. However, several novel vaccine candidates have been tested in mouse models. A summary of the different types of vaccines in development was recently presented at the 11th International *Bordetella* symposium [55].

5.1. Whole-cell vaccines with reduced endotoxin contents

Considering that most of the adverse events observed after DTP vaccination may be due to the high endotoxin or lipooligosaccharide (LOS) content of whole-cell vaccines, efforts have been deployed to reduce the LOS content of these vaccines or to modify their endotoxic activity. However, LOS also expresses strong immune stimulating activity and therefore may contribute to the efficacy of the whole-cell vaccines. Recombinant *B. pertussis* strains that produce the LOS modifying enzyme PagL have been constructed and shown to increase vaccine efficacy in a mouse model. However, it did not significantly alter reactogenicity [56]. An alternative way is to decrease endotoxicity by chemical extraction/-modification of LOS form the outer membrane [57]. This was shown to indeed reduce reactogenicity, as measured by several *in vivo* and *in vitro* toxicity assays, without affecting potency, immunogenicity or the Th1/Th2 balance in a murine challenge model. A whole-cell vaccine with reduced endotoxin content has been compared with a classical whole-cell vaccine for immunogenicity and reactogenicity in a head-to-head clinical trial involving over 100 infants in each arm [58]. This trial showed comparable immunogenicity between the two types of vaccines, both at the antibody and at the T cell levels. However, there was also no significant difference in reactogenicity between the two types of vaccines, but the study was not powered to see potential differences in severe adverse events.

5.2. Outer membrane vesicles

An alternative to whole-cell vaccines are outer membrane vesicles (OMV). These vesicles are released from the *Bordetella* organisms and contain many antigenic proteins, including the major protective antigens, as well as LOS [59]. The safety profile, as evaluated in mouse models, appears to be comparable to that of acellular vaccines, and substantially better than that of whole-cell vaccines [60]. Combined with aluminium hydroxide, they are immunogenic in mice and able to induce protection against nasal challenge at a level that is comparable to that induced by a whole-cell vaccine [59]. Although not tested in human clinical trials yet, *B. pertussis* OMVs combined with diphtheria and tetanus toxoids showed low toxicity in an *in vitro* human whole blood assay [61].

5.3. Novel DTaP formulations and adjuvants

Improvements of the acellular vaccines have also been proposed. They include novel formulations, Th1- or Th17-driving adjuvants and the addition of new antigens. A formulation of acellular pertussis vaccine in microparticles containing polyphosphazene, cationic innate defence regulator peptide and CpG oligodeoxynucleotides was found to induce earlier onset of immunity and higher levels of anti-*B. pertussis* IgG2a and IgA in mice than a commercial acellular pertussis vaccine [62]. Similarly, when DTaP vaccines were adsorbed onto negatively charged poly(lactide-co-glycolide) nanoparticles that were combined with a synthetic TLR-7 ligand, IgG and IgG2a levels in mice were enhanced, provided that the TLR-7 ligand was co-localized with the antigens within the same nanoparticle [63]. An increase in antigen-specific IFN-γ and IL-17 secretion by splenocytes from mice immunized with pertussis toxoid incorporated in poly(lactic-co-glycolic acid)-based nano/micro particles was observed compared to splenocytes from mice vaccinated with soluble pertussis toxoid, although the IgG levels were similar in both mouse groups [64]. The importance of Th1 cytokines, such as IFN-γ, and Th17 cytokines in protection against lung infection by *B. pertussis* in murine models has been elegantly demonstrated by the finding that acellular pertussis vaccines fail to protect IL-17A-defective mice against challenge, and that optimal protection against *B. pertussis* challenge also requires the induction of Th1 cells [65]. Therefore, the combination of DTaP and Tdap with adjuvants that are able to drive Th1 and Th17 responses would be expected to enhance protection. The intranasal administration of cyclic di-GMP to mice has been shown to enhance Th1 type immune responses to infection with *B. pertussis*, to enhance protective innate immunity and to significantly reduce the bacterial burden in the lungs of the infected mice [66]. However, cyclic di-GMP has not yet been evaluated as an adjuvant combined with DTaP or Tdap. Adjuvants that are already approved for human use, such as MF59 emulsions and the combination of aluminium hydroxide with the TLR-4 agonist monophosphoryl lipid A, both strong inducers of Th1 immune responses, have also been shown to lead to a more rapid onset of antibody responses and to a change in antibody quality [67]. It has not been reported whether these combinations also lead to improved protection in the mouse model. The *B. pertussis* lipoprotein BP1569 has recently been shown to possess in-built adjuvant properties of whole-cell extracts [68]. This protein is a TLR-2 agonist and activates murine dendritic cells and macrophages, as well as human mononuclear cells. A synthetic lipopetide corresponding to the N-terminal portion of BP1569 was found to enhance Th1, Th17 and IgG2a responses in mice when combined with acellular pertussis vaccine, and this formulation conferred superior protection against *B. pertussis* challenge than the same vaccine without the lipopeptide. Altogether, these study show that novel formulations and adju-

vants are able to ameliorate the immune responses to acellular pertussis vaccines. Whether this translates into longer lived protection or to prevention of *B. pertussis* infection and transmission in humans remains to be evaluated.

5.4. Novel vaccine antigens

In addition to novel formulations and adjuvants, new antigens have also been explored. The *B. pertussis* adenylate cyclase toxin has been proposed as a protective antigen more than 30 years ago [69]. It is immunogenic in humans, as patients diagnosed with pertussis readily mount antibodies to this antigen [70]. Various recombinant and genetically inactivated forms of adenylate cyclase toxin have also been tested in mouse protection assays. In one study, none of these forms offered significant protection against *B. pertussis* infection in an intranasal challenge model [71]. However, when combined with acellular pertussis vaccine, some forms showed conferred improved protection over the acellular vaccine alone.

The serum-resistance autotransporter protein BrkA has also been evaluated as a protective antigen in murine challenge studies. Antibodies raised to the passenger domain of BrkA can neutralize the serum-resistance properties of this protein and augment the bactericidal capacity of human serum [72]. Therefore, this protein may be an interesting candidate to add to acellular pertussis formulations. BrkA alone did not significantly protect mice against *B. pertussis* challenge, but the addition of BrkA to a DTaP vaccine containing only PT and FHA as the sole *B. pertussis* antigens was found to increase protection against lung colonization upon nasal infection [73]. However, when it was combined to a DTaP that contains pertactin in addition to PT and FHA, no further protective efficacy was observed. These findings suggest that pertactin may be replaced by BrkA, which may be interesting to consider in the context of the expansion of pertactin-deficient *B. pertussis* strains [22].

Iron-regulated *B. pertussis* proteins are also attractive vaccine candidates, as their production is induced by iron starvation, a condition that is encountered during infection. *B. pertussis*-infected individuals mount an antibody response to several iron-regulated proteins, especially to the protein called IRP1-3, and vaccination of mice with this protein conferred protection against *B. pertussis* challenge [74]. Similarly a second highly antigenic iron-regulated protein, named AfuA, also induced protection against *B. pertussis* infection in mice [75]. Both proteins are well conserved among clinical isolates. Interestingly, the addition of both proteins to commercial DTaP vaccine significantly increased the protective activity of the vaccine in mice.

The major surface antigen of *B. pertussis* is LOS. However, the *B. pertussis* LOS is poorly immunogenic, in comparison to lipopolysaccharide molecules of other Gram negative organisms. Nevertheless serum antibodies to LOS are induced in children infected with *B. pertussis*, in contrast to children vaccinated with either DTP or DTaP [76]. When the core oligosaccharide portion of the *Bordetella* LOS was conjugated to the carrier protein bovine serum albumin, it induced bactericidal antibodies in mice that were able to kill *B. pertussis* in a complement-dependent fashion [77]. However, so far the protective effect of this vaccine candidate has not been assessed in any animal model.

Proteomic technologies have also bee exploited to identify potential vaccine antigens for pertussis. Using this approach several proteins were identified that were produced by *B. pertussis* during infection of the mouse lungs. Two of them, Vag8 and SphB1, induced significant opsonizing antibody responses and protection against lung colonization in mice [78]. Whether they increase the protective effect of DTaP if added to this vaccine is not yet known.

5.5. Live attenuated vaccines

Based on the concept that asymptomatic infection and transmission may be a major driver of the pertussis resurgence [28], that, in contrast to prior infection, current pertussis vaccines do not prevent asymptomatic infection [25] and that infection-induced protection is longer lived than vaccine-induced protection [79], an entirely different approach to the pertussis problem is the development of live attenuated vaccines in order to mimic infection without causing disease.

5.5.1. B. pertussis aro mutants

The first live pertussis vaccine candidate was based on the notion, successfully applied to other live attenuated vaccines, such as *Salmonella*, that *aroA* auxotrophs are strongly attenuated. An *aroA* mutant of *B. pertussis* was indeed strongly attenuated in a murine infection model and could induce anti-*B. pertussis* antibodies [80]. Repeated administrations of this strain also resulted in significant protection against virulent *B. pertussis* challenge in mice. More recently, another attenuated vaccine candidate was constructed by genetically deleting the *aroQ* gene, another gene involved in the aromatic amino acid synthesis pathway [81]. This strain persisted for 2 and 11 days in the mouse trachea and lungs, respectively, which was longer than the persistence of the *aroA* mutant. Consequently, antibodies to *B. pertussis* antigens and significant protection was already seen after a single nasal administration of the *aroQ* mutant.

5.5.2. The development of BPZE1

The most advanced live pertussis vaccine candidate is BPZE1 [82]. This *B. pertussis* strain was constructed by the genetic inactivation or removal of three major toxins. The structural gene coding for the dermonecrotic toxin was deleted. The *B. pertussis ampG* gene was replaced by the *ampG* gene of *Escherichia coli*, thereby reducing the production of the tracheal cytotoxin to background levels. Finally, two codons of the PT S1 subunit gene were modified. Each of the two modifications abolished the enzymatic ADP-ribosyltransferase activity of the toxin and thereby reduced the toxic activity of PT to undetectable levels. Thus BPZE1 produces and secretes an antigenically intact, yet genetically detoxified PT analogue [83]. These genetic modifications did not affect the size or shape of *B. pertussis*, nor did they modify the *in vitro* growth characteristics or binding capacity to host cells. Whole genome sequencing of BPZE1 showed that beside the above changes, this strain was isogenic with its virulent parent strain. The mouse lung colonization capacity of BPZE1 was similar to that of its parent strain, with persistence for 3–4 weeks, although the peak of multiplication seen 7 days after infection with the virulent strain was lacking with the vaccine strain. Despite persistent colonization, BPZE1 did not induce histopathological changes and inflammatory infiltrations in the lungs of 8-week old or 3-week old mice, in contrast to the virulent parent strain.

5.5.3. BPZE1-induced protection against B. pertussis challenge in animal models

A single nasal administration of 10^6 colony-forming units (CFU) of BPZE1 induced anti-*B. pertussis* serum antibodies at levels comparable to those induced by two intraperitoneal administrations of 1/5 of a human DTaP vaccine [83]. However, the isotype profiles between the two vaccine groups were very different. Whereas DTaP induced essentially IgG1, BPZE1 induced mostly IgG2a to FHA and PT. This was paralleled by the induction of strong Th1 type cytokines by the live vaccine, in contrast to the Th2 type cytokines induced by DTaP. Adult, 8-week old mice were protected against *B. challenge* in the mouse lung colonization model after a single nasal administration of BPZE1, at levels similar to protection

seen after two 1/5 human dose of DTaP. However, in infant mice, 2 injections of DTaP protected poorly against lung colonization by virulent *B. pertussis*, whereas a single nasal administration of BPZE1 resulted in complete bacterial clearance in the lungs one week after challenge infection. Protection in mice is dose-dependent. Although BPZE1 persistence is not strongly dependent on the dose, and lung colonization usually lasts for 3–4 weeks if mice are infected with different doses ranging from 10^3 to 10^6 CFU, the overall bacterial load directly depends on the infectious dose. Anti-*B. pertussis* antibody titers, antigen-specific IFN-γ production and CFU reduction upon virulent *B. pertussis* challenge were directly correlated with the vaccine dose [84].

Protection against lung infection is mediated by both serum antibodies and by $CD4^+$ T cells. Serum from BPZE1-vaccinated mice is able to kill *B. pertussis* organisms *in vitro* [85]. Passive transfer of either serum or splenocytes from BPZE1-vaccinated mice to SCID mice protects the SCID mice form lung infection by nasally administered virulent *B. pertussis* [85]. When the splenocytes were sorted for $CD4^+$ and $CD8^+$ T cells, only the $CD4^+$ T cells could transfer BPZE1-induced protection to the SCID mice, suggesting that the $CD4^+$ T cells are the major effector T cells in protection against lung infection. However, although no protective effect of $CD8^+$ T cells was observed after passive transfer to the SCID mice, it cannot be ruled out that these cells may also contribute to protection if they require $CD4^+$ T cell help to exert their effector function.

BPZE1-induced protection against *B. pertussis* challenge is long lived. When adult or infant mice were vaccinated with a single nasal dose of BPZE1 and challenged at different time intervals with virulent *B. pertussis*, full protection against lung colonization was still observed one year after vaccination, independent of the age of the mice at vaccination [86,87]. In contrast, waning of immunity induced by two administrations of 1/5 of the human DTaP dose was observed at 12 months after vaccination for the adult mice and already at 6 months after vaccination for the infant mice. One year after vaccination high titers of anti-*B. pertussis* serum antibodies and high levels of *B. pertussis*-specific memory T cells were still present in the BPZE1-treated mice. Furthermore, serum and splenocytes from BPZE1-vaccinated mice harvested one year after vaccination still passively transferred protection against *B. pertussis* challenge to SCID mice, whereas this was not the case for serum or splenocytes of DTaP-vaccinated mice.

Priming of neonatal or infant mice with BPZE1, followed by two doses of DTaP was found to enhance antibody levels to the *B. pertussis* antigens, especially IgG2a, whereas the *B. pertussis*-specific IgG1 titers were not affected or decreased by BPZE1 priming [88]. This was paralleled by the cytokine profile, in which BPZE1 priming resulted in elevated Th1 and Th17 cytokine levels upon DTaP boosting, whereas the Th2 cytokine levels were reduced by the BPZE1 priming. Thus, BPZE1 priming causes persistence of the Th1/Th17 type responses after DTaP vaccination, which otherwise gears the immune response towards a Th2 type. Priming with low doses of BPZE1 also strongly augmented the protection mediated by DTaP in the murine nasal challenge model.

More recently, BPZE1 has also been tested in non-human primates [89]. Administration of 10^9 or 10^{10} CFU of BPZE1 to baboons protected these animals against pertussis disease and leukocytosis upon challenge with a high dose (>10^{10} CFU) of a highly virulent recent clinical isolate, which killed one out of three non-vaccinated baboons. In addition to fully protecting against pertussis disease BPZE1 also strongly affected colonization by the virulent strain, which was reduced by 99.998%, compared to the non-vaccinated animals.

5.5.4. Heterologous protection by BPZE1

Interestingly, BPZE1 also protects mice against lung infection by *Bordetella parapertussis*, whereas this was not seen after vaccination with DTaP [83,85]. However, in this case, protection could only be transferred to SCID mice by spenocytes from BPZE1-vaccinated mice. The transfer of serum from the vaccinated mice did not result in any significant protection of the SCID mice against *B. parapertussis* infection [85]. Furthermore, serum from BPZE1-vaccinated mice failed to kill *B. parapertussis in vitro*, whereas the same anti-serum could effectively kill *B. pertussis*.

Subsequent studies revealed that BPZE1 also protects against *Bordetella bronchiseptica* [90]. In a murine lethal challenge model BPZE1 was found to reduce both lung colonization by *B. bronchiseptica* and *B. bronchiseptica*-induced death. These two protective effects relied on two different mechanisms. Reduction of CFUs in the lungs depended on adaptive T-cell immunity, whereas protection against mortality was mainly due to potent anti-inflammatory properties of BPZE1. These anti-inflammatory activities were reduced when anti-CD25 antibodies were injected to deplete $CD4^+$-$CD25^+FoxP3^+$ regulatory T cells.

The anti-inflammatory properties of BPZE1 have also been observed in other infection models, such as influenza A [91] and respiratory syncytial virus (RSV) infection [92]. A single nasal administration of BPZE1 significantly reduced mortality induced by a highly virulent mouse-adapted Influenza A strain [91]. Protection was dose-dependent. It was maintained for at least 12 weeks after vaccination and required live BPZE1. Heat-inactivated BPZE1 given nasally did not prevent virus-induced death of the mice. However, BPZE1 vaccination did not prevent viral infection, nor did it reduce viral load, and no cross-reactivity at the B- or T-cell level could be detected between BPZE1 and Influenza virus A. Instead, BPZE1 vaccination substantially reduced the lung immunopathology, protected against lymphocyte depletion and dampened the inflammatory cytokine storm induced by the virus. Interestingly, a second administration of BPZE1 increased the survival rate of the influenza-infected mice and further reduced the inflammatory cytokine levels. As this protective mechanism does not rely on adaptive T- or B-cell-mediated immunity these observations suggest that BPZE1 induces some kind of "trained innate immunity" [93].

Similarly, in a murine model of RSV infection, nasal administration of BPZE1 completely abolished the weight loss induced by RSV [92]. It also reduced the cell numbers recruited to the airway after RSV infection and altered lung inflammation. Importantly, neonatal vaccination with BPZE1 resulted in long-lasting protection against RSV disease, as a single nasal BPZE1 dose given on days 2–5 of life prevented RSV-induced weight loss in adulthood. Flow cytometry analyses revealed that BPZE1 priming increased the numbers of IL-10-producing $CD4^+$ T cells in the lungs of RSV-infected mice, as well as the numbers of IL-17-producing $CD4^+$ T cells. Neutralization by blocking anti-IL-17 antibodies re-established the RSV-induced weight loss, but did not augment inflammation. It did, however, impair the recruitment of IFN-γ/IL-10 double producing $CD4^+$ and $CD8^+$ T cells.

In addition to virus-induced lung inflammation, BPZE1 also prevents inflammation caused by other aetiologies. In an experimental ovalbumin-induced murine asthma model, nasal administration of BPZE1 before ovalbumin sensitization resulted in decreased peribronchial inflammation upon ovalbumin challenge, compared to what was seen in sensitized mice in the absence of BPZE1 [94]. Mucus secretion from goblet cells was also decreased in BPZE1-treated mice, as were eosinophil influx into the lungs and the levels of Th2 cytokines in broncho-alveolar lavage fluids. Similar observations were made when the mice were vaccinated with BPZE1 6 weeks before sensitization [95]. Although BPZE1 treatment significantly reduced Th2 cytokine production it did not affect the production of the Th1 cytokine IFN-γ. To determine whether the anti-inflammatory effect of BPZE1 was restricted to Th2-mediated inflammatory disorders, its protective effect was also

evaluated in a contact hypersensitivity model, a Th1-driven inflammatory condition. A single nasal administration of BPZE1 did not modify the ear swelling of mice sensitized and then challenged with dinitrochlorobenzene. However, two doses of BPZE1, given 4 weeks apart, resulted in a signification inhibition of ear swelling in dinitrochlorobenzene treated mice, with less tissue edema and inflammatory infiltrate. Two administrations of BPZE1 also reduced the levels of pro-inflammatory cytokines in the ear homogenates. Thus, BPZE1 mediates protection against both Th2- and Th1-mediated inflammatory disorders, whether in the lungs or at distal sites.

Although the mechanism of the anti-inflammatory activities of BPZE1 are not yet elucidated, it is tempting to speculate that both $CD4^+CD25^+FoxP3^+$ Treg cells and IL-10-producing $CD4^+$ T cells may contribute, perhaps in a synergistic manner, and both may depend on IL-17. However, this requires further investigation. *In vitro* work on human dendritic cells has shown that BPZE1 may also drive Th1/Th17 responses in humans [96]. BPZE1 was found to activate human dendritic cells and to induce the production of a broad range of pro-inflammatory and regulatory cytokines, and BPZE1-primed dendritic cells can very efficiently migrate *in vitro* in response to chemokines and drive a mixed Th1/Th17 polarization. A more recent study has shown that BPZE1-exposed dendritic cells induce T lymphocytes to express CD39/CD73, which uses ATP as a substrate to produce adenosine, and CD38/CD203a/CD73, which uses NAD^+ as a substrate to also produce adenosine [97]. This results in a regulatory phenotype, which may also be a potential mechanism of the anti-inflammatory activity of BPZE1.

Importantly, the anti-inflammatory properties of BPZE1 do not result in immune suppression, as antibody levels or antigen-specific T cell responses to viral or bacterial antigens are not altered by BPZE1 administration, nor does BPZE1 increase the bacterial [83] or viral load [91,92] upon heterologous infection.

5.5.5. Safety and stability of BPZE1

Important issues of live vaccines intended for large-scale vaccination programs are their safety and genetic stability. The initial work in mice has shown that, in contrast to its virulent parent strain, BPZE1 does not induce lung pathology after nasal administration, neither in adult, nor in infant mice [83]. In contrast to virulent *B. pertussis* it also does not predispose mice to other respiratory infections [83,91,92]. Nasal administration of BPZE1 to infant mice also does not affect the weight gain of these mice, nor does it result in any clinical symptoms [84]. In baboons, administration of up to 10^{10} CFU of BPZE1 resulted in no clinical sign of disease, the baboons did not cough, showed no increase in white blood cell counts (a hallmark of pertussis) and no physical abnormalities [89]. *In vitro* assays on human pulmonary epithelial cells or monocytic cells did also not show any toxicity of BPZE1 on these cells.

Whereas the virulent parent strain was able to kill neonatal mice in a dose-dependent manner, no mortality was observed when neonatal mice were infected with up to 10^6 CFU of BPZE1, the highest dose tested [98]. A similar safety profile was also observed in immune deficient mice, such as IFN-γ-receptor-deficient mice [98]. Although *B. pertussis* is a strictly mucosal pathogen, restricted to the respiratory tract, rare disseminated *B. pertussis* infection has been described in highly immune compromised individuals [99]. Atypical, disseminated infection can also be observed in IFN-γ-receptor-deficient mice. However, whereas the virulent parent strain caused atypical disseminated disease in these mice, this was not the case for BPZE1 [98]. Both strains colonized the lungs of these mice equally well, but no dissemination to the liver, spleen or blood was observed for BPZE1. In contrast, the virulent could readily be detected in the liver 7 days after administration.

The genetic stability of BPZE1 was assessed both after serial passages *in vitro* and *in vivo* [100]. BPZE1 was grown on blood agar medium and serially passaged once a week, for a total of 20 passages. In parallel, BPZE1 was nasally administered to adult mice. Their lungs were harvested two weeks later, and the *Bordetella* organisms present in the lungs were cultured for one week on blood agar medium, after which they were harvested and used again to infect adult mice. This was repeated for nine cycles. After 20 passages *in vitro* and 9 passages *in vivo*, the resulting bacteria were analysed for the presence of the expected genetic alterations. All bacteria were found to have the expected deletion of the dermonecrotic toxin gene, the replacement of the *B. pertussis ampG* gene by that of *E. coli* and the expected codon changes in the PT S1 subunit gene. Furthermore, full genome DNA arrays were used to confirm that no other major genetic change had occurred during the various passages. Finally, the potency of the passaged strain was compared to that of the original BPZE1 suspension in the mouse respiratory challenge model, which indicated that neither the *in vitro* nor the *in vivo* passages had affected the potency of BPZE1.

These results were expected, as by virtue of the nature of the genetic modifications that led to the generation of BPZE1, a reversion to virulence can be considered impossible. Entire gene deletions are impossible to revert. Either one of the codon changes that led to PT inactivation detoxifies PT independently. One of the codons has resulted in the elimination of a catalytic residue of PT, and the second codon change abolished the substrate binding activity of PT [83]. Thus even in the unlikely event that one codon would revert, the second codon change would still abolish toxin activity. Furthermore, the genome of *B. pertussis* contains no genetic information for a horizontal gene transfer system [101], which makes horizontal transfer of virulence determinants from a wild-type *B. pertussis* strain to BPZE1 virtually impossible.

5.5.6. Clinical development of BPZE1

The excellent pre-clinical safety profile together with the genetic stability of BPZE1 has allowed this strain to be downgraded from a safety level 2 to a safety level 1 organism in several countries. This has made its clinical development possible. A first-in-man clinical trial with BPZE1 has been successfully completed and shown that BPZE1 is safe in adult male volunteers, able to transiently colonize the human respiratory tract and to induce immune responses in the colonized individuals [102]. This study was conducted in Sweden, a country in which pertussis vaccination had come to a full stop between 1979 and 1991. All volunteers included in the study were born in that period of time and never had a pertussis vaccination.

The vaccine was given in each nostril as a 100 μl nasal drop suspension containing either 10^3, 10^5 or 10^7 CFU of BPZE1 to young male volunteers who had less than 20 international units (IU) of anti-PT serum antibodies. The trial was double blind and placebo controlled, and each vaccine group was composed of 12 subjects receiving the vaccine and 4 subjects receiving placebo. Thus, together the trial comprised 12 placebo controls equally distributed throughout the three vaccination groups. The study subjects were followed up for 6 months. There was no vaccine-related severe adverse event in any of the dosage groups. All adverse events were all trivial and short-lasting, and there was no difference between the placebo group and any of the treatment groups.

BPZE1 bacteria could be isolated from the nasopharyngeal aspirates of one out of 12 subjects of the low and intermediate dose group, and of 5 individuals out of 12 of the high (10^7 CFU) group. Bacterial colonization was transient and lasted for a maximum of about 2 weeks. Serum antibodies to PT, FHA, pertactin and fimbriae appeared in the colonized subjects between 14 and 28 days and

remained for at least up to 6 months after vaccination at levels that were as high as at one month. No antibody levels above baseline were detected in the non-colonized volunteers, indicating that bacterial colonization was essential for the induction of anti-*B. pertussis* antibodies. Antigen-specific plasmablasts could also readily be detected in the blood from colonized subjects, but not from the non-colonized individuals [103]. Their numbers increased between 7 and 14 days after vaccination. Similarly, the culture positive volunteers had significant increases in antigen-specific memory B cells, in particular activated memory B cells, between day 0 and day 28. For most subjects the memory B cell population had decreased again at 6 months after vaccination, most likely because they had homed to secondary lymphoid organs.

However, even with the highest dose tested in this trial, only approximately 40% of the subjects were colonized and induced anti-*B. pertussis* antibodies [102]. Several reasons might account for this relatively low vaccine take. Perhaps even 10^7 CFU may not be a sufficiently high dose for optimal vaccine take. Furthermore, 100 μl/nostril may be a volume too small to effectively cover the mucosal surface of the nasal cavity. Thirdly, an interesting observation was made when the pre-existing antibodies were measured in the colonized and the non-colonized subjects. Pre-existing antibody levels to FHA, pertactin and fimbriae were significantly higher in the non-colonized group, as compared to the colonized group. This was not the case for anti-PT antibodies, as volunteers with high pre-existing anti-PT titers were excluded from the study. Since the study subjects had never received a pertussis vaccination before BPZE1 administration, the antibodies detected against FHA, pertactin and fimbriae are most likely the result of prior silent *Bordetella* infection, as individuals that had experienced overt pertussis disease were excluded from the study. It is well known that serum antibodies to these adhesins are longer lived than antibodies to PT, which wane one to two years after *B. pertussis* infection. Among the 85 volunteers who were screened for anti-PT antibodies, 22 had levels above 20 IU and were therefore not included in the study. This suggests that roughly 25% of the study subjects had experienced silent *B. pertussis* infection within the previous 2 years. Therefore, it is likely that the prior infection by circulating *B. pertussis* may have induced protective immunity that prevented BPZE1 take.

To counter the various reasons for the relatively poor vaccine take in this phase I trial, another trial is currently ongoing in which BPZE1 is nasally delivered in a higher volume (400 μl/nostril). The dose is escalated from 10^7, to 10^8, to 10^9 CFU, and subjects with high pre-existing antibodies to pertactin or to PT have been excluded. It is hoped that these modifications will lead to an improved vaccine take.

6. Conclusion

Despite high vaccination coverage, pertussis has made a recent spectacular come-back in several countries, especially in those countries where DTP was replace by DTaP. Several strategies have been recommended to cope with the pertussis problem. They include frequent booster vaccinations with Tdap, cocoon vaccination of the close contacts to a new-born child, and, more recently, maternal vaccination with Tdap during the third trimester of each pregnancy. All three strategies have their inherent difficulties for large-scale implementation. Whereas the first two strategies did not result in an effective control of whooping cough, maternal immunization has shown promise in several independent studies to significantly decrease severe pertussis disease in neonates. However, none of these strategies is likely to result in the ultimate control of pertussis, as neither DTP, nor DTaP or Tdap prevent asymptomatic infection and silent transmission of the pathogen. Therefore, novel vaccines are needed. Various options are being explored and have shown promise in pre-clinical mouse models. Several of them are based on the increased knowledge on pertussis immunity gathered over recent years. In particular the role of Th-1 and Th-17 type T cell responses, in addition to antibodies, is now well recognized and has been the basis of pre-clinical developments of vaccine candidates formulated with novel adjuvants. However, it is also likely that mucosal immunity, including IL-17-producing tissue-resident memory T cells [104], plays an important role in protection against pertussis, as *B. pertussis* is a strictly mucosal pathogen. The most advanced novel pertussis vaccine is the live attenuated *B. pertussis* strain BPZE1, which is designed to be administered by the nasal route in order to mimic natural infection, known to prevent subsequent infection. However, even the live attenuated vaccine is still in the early phases of clinical development. Whether it will eventually successfully complete all subsequent clinical studies, including efficacy trials, and reach the market remains of course to be seen. However it is today the only new pertussis vaccine candidate in development that is specifically aimed at reducing carriage and transmission of *B. pertussis*, which would be a major step towards the ultimate control of the disease. Furthermore, its predicted low production cost should make it affordable for all countries in the world. Whether we will have a new vaccine like this also strongly depends on the financial resources that can be devoted to the development of such a vaccine.

References

[1] Condit JC. Results from pertussis vaccine. Cal State J Med 1918;16:529.
[2] Medical Research Council. The prevention of whooping cough by vaccination. Br Med J 1951;1:1463–71.
[3] Fine PEM, Clarkson JA. Reflections on the efficacy of pertussis vaccines. Rev Infect Dis 1987;9:866–83.
[4] Cody CL, Baraff LJ, Cherry JD, Marcy SM, Manclark CR. Nature and rates of adverse reactions associate with DTP and DT immunizations in infants and children. Pediatrics 1981;68:650–60.
[5] Romanus V, Jonsell R, Bergquist SO. Pertussis in Sweden after the cessation of general immunization in 1979. Pediatr Infect Dis J 1987;6:664–71.
[6] **Sato Y, Kimura M, Fukumi H. Development of a pertussis component vaccine in Japan. Lancet 1984;i:122–6.**
[7] **Ad hoc group for the study of pertussis vaccines. Placebo-controlled trial of two acellular pertussis vaccines in Sweden – protective efficacy and adverse events. Lancet 1988;i:955–60.**
[8] Gustafsson L, Hallander HO, Olin P, Reizenstein E, Storsaeter J. A controlled trial of a two-component acellular, a five-component acellular, and a whole-cell pertussis vaccine. N Engl J Med 1996;334:349–55.
[9] Greco D, Salmaso S, Mastrantonio P, Giuliano M, Tozzi AE, Anemona A, et al. A controlled trial of two acellular vaccines and one whole-cell vaccine against pertussis. N Engl J Med 1996;334:341–8.
[10] Simondon F, Preziosi MP, Yam A, Kane CT, Chabirand L, Iteman I, et al. A randomized double-blind trial comparing a two-component acellular to a whole-cell pertussis vaccine in Senegal. Vaccine 1997;15:1606–12.
[11] Rohani P, Zhong X, King AA. Contact network structure explains the changing epidemiology or pertussis. Science 2010;330:982–5.
[12] Cherry JD. Epidemic pertussis in 2012 – the resurgence of a vaccine-preventable disease. N Engl J Med 2012;367:785–7.
[13] Amirthalingam G, Gupta S, Campbell H. Pertussis immunisation and control in England and Wales, 1957 to 2012: a historical review. Euro Surveill 2013;2013(18):1–9.
[14] Sizaire V, Garrido-Espeta M, Masa-Calles J, Martinez de Aragon MV. Increase of pertussis in 2010 to 2012 after 12 years of low circulation in Spain. Euro Surveill 2014;2014(19):1–8.
[15] Jogi P, Oona M, Toompere K, Leedo S, Epstein J, Lustar I. Seroprevalence of IgG antibodies to pertussis toxin in children and adolescents in Estonia. Vaccine 2014;32:5311–5.
[16] Tan T, Dalby T, Forsythe K, Halperin SA, Heininger U, Hozbor D, et al. Pertussis across the globe. Pediatr Infect Dis J 2015;34:e222–32.
[17] Sheridan SL, Ware RS, Grimwood K, Lambert SB. Number and order of whole cell pertussis vaccines in infancy and disease protection. JAMA 2012;308:454–6.
[18] Witt MA, Katz PH, Witt DJ. Unexpectedly limited durability of immunity following acellular pertussis vaccination in preadolescents in a North American outbreak. Clin Infect Dis 2012;54:1730–5.
[19] Burns DL, Meade BD, Messionnier NE. Pertussis resurgence: perspectives from the working group meeting on pertussis on the causes, possible paths forward, and gaps in our knowledge. J Infect Dis 2014;209(S1):S32–5.

[20] Mooi FR. *Bordetella pertussis* and vaccination: the persistence of a genetically monomorphic pathogen. Infect Genet Evol 2010;10:36–49.

[21] Van Gent M, Heuvelman CJ, van der Heide HG, Hallander HO, Advani A, Guiso N, et al. Analysis of *Bordetella pertussis* clinical isolates circulating in European countries during the period 1998–2012. Eur J Clin Microbiol Infect Dis 2015;34:821–30.

[22] Martin SW, Pawloski L, Williams M, Weening K, DeBolt C, Qin X, et al. Pertactin-negative *Bordetella pertussis* strains: evidence for a possible selective advantage. Clin Infect Dis 2015;60:223–7.

[23] Vodzak J, Queenan AM, Souder E, Evangelista AT, Long SS. Clinical manifestations and molecular characterization of pertactin-deficient and pertactin-producing *Bordetella pertussis* in children, Philadelphia 2007–2014. Clin Intfec Dis 2017;64:60–6.

[24] Safarchi A, Octavia S, Luu LD, Tay CY, Sintchenko V, Wood N, et al. Pertactin negative *Bordetella pertussis* demonstrates higher fitness under vaccine selection pressure in a mixed infection model. Vaccine 2015;33:6277–81.

[25] Warfel JM, Zimmerman LI, Merkel TJ. Acellular pertussis vaccines protect against disease but fail to prevent infection and transmission in a nonhuman primate model. Proc Natl Acad Sci USA 2014;111:787–92.

[26] Fine PE, Clarkson JA. The recurrence of whooping cough: possible implications for assessment of vaccine efficacy. Lancet 1982;319:666–9.

[27] Rohani P, Earn DJ, Grenfell BT. Impact of immunization on pertussis transmission in England and Wales. Lancet 2000;355:285–6.

[28] Althouse BM. Scarpino. Asymptomatic transmission and the resurgence of *Bordetella pertussis*. BMC Med 2015;13:146.

[29] Dagan R, Igbaria K, Piglansky L, Van Brusteghem F, Melot V, Kaufhold A. Reactogenicity and immunogenicity of reduced antigen content diphtheria-tetanus-acellular pertussis vaccines as a booster in 4-7-year-old children primed with diphtheria-tetanus-whole cell pertussis vaccine before 2 years of age. Vaccine 1999;17:2620–7.

[30] Scheifele DW, Halerpin SA, Ochnio JJ, Ferguson AC, Skowronski DM. A modified vaccine reduces the rate of large injection site reactions to the preschool booster dose of diphtheria-tetanus-acellular pertussis vaccine: results of a randomized, controlled trial. Pediatr Infect Dis J 2005;24:1059–66.

[31] **Broder KR, Corteses MM, Iskander JK, Kretsinger K, Slade BA, Brown KH, et al. Preventing tetanus, diphtheria, and pertussis among adolescents: use of tetanus toxoid, reduced diphtheria toxoid and acellular pertussis vaccines recommendations of the Advisory Committee on Immunization Practices (ACIP)? MMWR Recomm Rep 2006;55(RR-3):1-34.**

[32] Centers fo Disease Control and Prevention. CDC). Updated recommendations for use of tetanus toxoid reduced diphtheria toxoid, and acellular pertussis (Tdap) vaccine in adults aged 65 years and older: Advisory Committee on Immunization Practices (ACIP). MMWR Morb Mortal Wkly Rep 2012;61:468–70.

[33] Southern J, Waight PA, Andrews N, Miller E. Extensive swelling of the limb and systemic symptoms after a fourth dose of acellular pertussis containing vaccines in England in children aged 3–6 years. Vaccine 2017;35:619–25.

[34] Baxter R, Lewis E, Goddard K, Fireman B, Bakshi N, DeStefano F, et al. Acute demyelinating events following vaccines: a case-centered analysis. Clin Infect Dis 2016;63:1456–62.

[35] Tomovici A, Barreto L, Zickler P, Meekison W, Noya F, Voloshen T, et al. Humoral immunity 10 years after booster immunization with an adolescent and adult formulation combined tetanus, diphtheria, and 5-component acellular pertussis vaccine. Vaccine 2012;30:2647–53.

[36] Terranella A, Rea V, Griffith M, Manning S, Sears S, Farmer A, et al. Vaccine effectiveness of tetanus toxoid, reduced diphtheria toxoid, and acellular pertussis vaccine during a pertussis outbreak in Main. Vaccine 2016;34:2496–500.

[37] Klein NP, Bartlett J, Fireman B, Baxter R. Waning Tdap effectiveness in adolescents. Pediatrics 2016;137:e20153326.

[38] Forsyth KD, Wirsing von König CH, Tan T, Caro J, Plotkin S. Prevention of pertussis: recommendations delivered from the second Global Pertussis Initiative roundtable meeting. Vaccine 2007;25:2634–42.

[39] Cohen R, Gaudelus J, Denis F, Stahl JP, Chevaillier O, Pujol P, et al. Pertussis vaccination coverage among French parents of infants after 10 years of cocoon strategy. Med Mal Infect 2016;46:188–93.

[40] Wiley KE, Zuo Y, Macartney KK, McIntyre PB. Sources of pertussis infection in young infants: a review of key evidence informing targeting of the cocoon strategy. Vaccine 2013;31:618–25.

[41] Urwyler P, Heininger U. Protecting newborns from pertussis – the challenge of complete cocooning. BMC Infect Dis 2014;14:397.

[42] Skowronski DM, Janjua NZ, Tsafack EP, Ouakki M, Hoang L, De Serres G. The number needed to vaccinate to prevent infant pertussis hospitalization and death through parent cocoon immunization. Clin Infect Dis 2012;54:318–27.

[43] Castagnini LA, Healy CM, Rench MA, Wootton SH, Munoz FM, Baker CL. Impact of maternal postpartum tetanus and diphtheria toxoids and acellular pertussis immunization on infant pertussis infection. Clin Infect Dis 2012;54:78–84.

[44] Healy CM, Rench MA, Wooton SH, Castagnini LA. Evaluation of the impact of a pertussis cocooning program on infant pertussis infection. Pediatr Infect Dis J 2015;43:22–6.

[45] Quinn HE, Snelling TL, Habig A, Chiu C, Spokes PJ, McIntyre PB. Parental Tdap boosters and infant pertussis: a case-control study. Pediatrics 2014;134:713–20.

[46] Carcione D, Regan AK, Tracey L, Mak DB, Gibbs R, Dowse GK, et al. The impact of parental postpartum pertussis vaccination on infection in infants: a population-based study of cocooning in Western Australia. Vaccine 2015;33:5654–61.

[47] Englund JA. The influence of maternal immunization on infant immune responses. J Comp Pathol 2007;137:S16–9.

[48] Munoz FM, Bond NH, Maccato M, Pinell P, Hammill HA, Swamy GK, et al. Safety and immunogenicity of tetanus diphtheria and acellular pertussis (Tdap) immunization during pregnancy in mothers and infants: a randomized clinical trial. JAMA 2014;311:1760–9.

[49] Amirthalingam G, Andrews N, Campbell H, Ribeiro S, Kara E, Donegan K, et al. Effectiveness of maternal pertussis vaccination in England: an observational study. Lancet 2014;384:1521–8.

[50] Winter K, Nickell S, Powell M, Harriman K. Effectiveness of prenatal versus postpartum tetanus, diphtheria, and acellular pertussis vaccination in preventing infant pertussis. Clin Infect Dis 2017;64:3–8.

[51] Healy CM. Pertussis vaccination in pregnancy. Hum Vaccin Immunother 2016;12:1972–81.

[52] Englund JA, Anderson EL, Reed GF, Decker MD, Edwards KM, Pichichero ME, et al. The effect of maternal antibody on the serologic response an the incidence of adverse reactions after primary immunization with acellular and whole-cell pertussis vaccines combined with diphtheria and tetanus toxoids. Pediatrics 1995;96:580–4.

[53] Maertens K, Caboré RN, Huygen K, Hens N, Van Damme P, Leuridan E. Pertussis vaccination during pregnancy in Belgium: results of a prospective controlled cohort study. Vaccine 2016;34:142–50.

[54] Feunou PF, Mielcarek N, Locht C. Reciprocal interference of maternal and infant immunization in protection against pertussis. Vaccine 2016;34:1062–9.

[55] Carbonetti NH, Wirsing von König CH, Lan R, Jacob-Dubuisson F, Cotter PA, Deora R, et al. Highlights of the 11th international *Bordetella* symposium; from basic biology to vaccine development. Clin Vaccine Immunol 2016;23:842–50.

[56] Geurtsen J, Vandebriel RJ, Gremmer ER, Kuipers B, Tommassen J, van der Ley P. Consequences of the expression of lipopolysaccharide-modifying enzymes for the efficacy and reactogenicity of whole-cell pertussis vaccines. Microbes Infect 2007;9:1096–103.

[57] Dias WO, van der Ark A, Sakauchi MA, Kubrusly FS, Prestes AF, Borges MM, et al. An improved whole cell pertussis vaccine with reduced content of endotoxin. Hum Vaccines Immunother 2012;9:339–48.

[58] Zorzeto TQ, Higashi HG, da Silva MT, Carniel E, Dias WO, Ramalho VD, et al. Immunogenicity of a whole-cell pertussis vaccine with low lipopolysaccharide content in infants. Clin Vaccine Immunol 2009;16:544–50.

[59] Roberts R, Moreni G, Bottero D, Gaillard ME, Fingermann M, Graieb A, et al. Outer membrane vesicles as acellular vaccine against pertussis. Vaccine 2008;26:4639–46.

[60] Rumbo M, Hozbor D. Development of improved pertussis vaccine. Hum Vaccin Immunother 2014;10:2450–3.

[61] Bottero D, Gaillard ME, Zurita E, Moreno G, Martinez DS, Bartel E, et al. Characterization of the immune response induced by pertussis OMVs-based vaccine. Vaccine 2016;34:3303–9.

[62] Polewicz M, Garcia A, Garlapati S, van Kessel J, Strom S, Halperin SA. Novel vaccine formulations against pertussis offer earlier onset of immunity and provide protection in the presence of maternal antibodies. Vaccine 2013;31:3148–55.

[63] Bruno C, Agnolon V, Berti F, Bufali S, O'Hagan DT, Baudner BC. The preparation and characterization of PLG nanoparticles with an entrapped synthetic TLR7 agonist and their preclinical evaluation as adjuvant for an adsorbed DTaP vaccine. Eur J Pharm Biopharm 2016;105:1–8.

[64] Li P, Asokanathan C, Liu F, Khaing KK, Kmiec D, Wei X, et al. PGLA nano/micro particles encapsulated with pertussis toxoid (PTd) enhances Th1/Th17 immune responses in a murine model. Int J Pharm 2016;513:183–90.

[65] Ross PJ, Sutton CE, Higgins S, Allen AC, Walsh K, Misiak A, et al. Relative contribution of Th1 and Th17 cells in adaptive immunity to *Bordetella pertussis*: towards the rational design of an improved acellular pertussis vaccine. PLoS Pathog 2013;6:e1003264.

[66] Elahi S, Van Kessel J, Kiros TG, Strom S, Hayakawa Y, Hyodo M, et al. C-di-GMP enhances protective innate immunity in a murine model of pertussis. PLoS One 2014;9:e109778.

[67] Agnolon V, Bruno C, Leuzzi R, Galletti B, D'Oro U, Pizza M, et al. The potential of adjuvants to improve immune responses against TdaP vaccines: a preclinical evaluation of MF59 and monophosphoryl lipid A. Int J Pharm 2015;492:169–76.

[68] Dunne A, Mielke LA, Allen AC, Sutton CE, Higgs R, Cunningham CC, et al. A novel TLR2 agonist from *Bordetella pertussis* is a potent adjuvant that promotes protective immunity with an acellular pertussis vaccine. Mucosal Immunol 2015;8:607–17.

[69] Novotny P, Chubb AP, Cownley K, Montaraz JA, Beesley JE. *Bordetella* adenylate cyclase: a genus specific protective antigen and virulence factor. Dev Biol Stand 1985;61:27–41.

[70] Arciniega JL, Hewlett EL, Johnson FD, Deforest A, Wassilak SG, Onorato IM, et al. Human serologic response to envelope-associated proteins and adenylate cyclase toxin of *Bordetella pertussis*. J Infect Dis 1991;163:135–42.

[71] Cheung GY, Xing D, Prior S, Corbel MJ, Parton R, Coote JG. Effect of different forms of adenylate cyclase toxin of *Bordetella pertussis* on protection afforded

by an acellular pertussis vaccine in a murine model. Infect Immun 2006;74:6797–805.
[72] Oliver DC, Fernandez RC. Antibodies to BrkA augment killing of *Bordetella pertussis*. Vaccine 2001;20:235–41.
[73] Marr N, Oliver DC, Laurent V, Poolman J, Denoel P, Fernandez RC. Protective activity of the *Bordetella pertussis* BrkA autotransporter in the murine lung colonization model. Vaccine 2008;26:4306–11.
[74] Alvarez Hayes J, Erben E, Lamberti Y, Ayala M, Maschi F, Carbone C, et al. Identification of a new protective antigen of *Bordetella pertussis*. Vaccine 2011;29:8731–9.
[75] Hayes J Alvarez, Erben E, Lamberti Y, Principi G, Maschi F, Ayala M, et al. *Bordetella pertussis* iron regulated proteins as potential vaccine components. Vaccine 2013;31:3543–8.
[76] Trollfors B, Lagergard T, Taranger J, Bergfors E, Schneerson R, Robbins JB. Serum immunoglobulin G antibody responses to *Bordetella pertussis* lipooligosaccharide and *B. parapertussis* lipopolysaccharide in children with pertussis and parapertussis. Clin Diagn Lab Immunol 2001;8:1015–7.
[77] Kubler-Kielb J, Vinogradov E, Lagergard T, Ginzberg A, King JD, Preston A, et al. Oligosaccharide conjugates of *Bordetella pertussis* and *bronchiseptica* induce bactericidal antibodies, an addition to pertussis vaccine. Proc Natl Acad Sci USA 2011;108:4087–92.
[78] De Gouw D, de Jonge MI, Hermans P, Wessels H, Zomer A, Berends A, et al. Proteomics-identified Bvg-activated autotransporters protect against *Bordetella pertussis* in a mouse model. PLoS One 2014;9:e105011.
[79] Wearing HJ, Rohani P. Estimating the duration of pertussis immunity using epidemiological signatures. PloS Pathog 2009;10:e1000647.
[80] Roberts M, Maskell D, Novotny P, Dougan G. Construction and characterization in vivo of *Bordetella pertussis aroA* mutants. Infect Immun 1990;58:732–9.
[81] Cornford-Nairns R, Daggard G, Mukkur T. Novel, non-reverting, intranasal live attenuated whooping cough vaccine candidate. J Microbiol Biotechnol 2012;22:856–65.
[82] Locht C, Mielcarek M. Live attenuated vaccines against pertussis. Exp Rev Vaccines 2014;13:1147–58.
[83] Mielcarek N, Debrie AS, Raze D, Bertout J, Rouanet C, Ben Younes A, et al. Live attenuated *B. pertussis* as a single-dose nasal vaccine against whooping couth. PLoS Pathog 2006;2:e65.
[84] Mielcarek N, Debrie AS, Mahieux S, Locht C. Dose response of attenuated *Bordetella pertussis* BPZE1-induced protection in mice. Clin Vaccine Immunol 2010;17:317–24.
[85] Feunou PF, Bertout J, Locht C. T- and B-cell-mediated protection induced by novel, live attenuated pertussis vaccine in mice. Cross protection against parapertussis. PLoS One 2010;5:e10178.
[86] Feunou PF, Kammoun H, Debrie AS, Mielcarek N, Locht C. Long-term immunity against pertussis induced by a single nasal administration of live attenuated *B. pertussis* BPZE1. Vaccine 2010;28:7047–53.
[87] Skerry CM, Mahon BP. A live attenuated *Bordetella pertussis* vaccine provides long-term protection against virulent challenge in a murine model. Clin Vaccine Immunol 2011;18:187–93.
[88] Feunou PF, Kammoun H, Debrie AS, Locht C. Heterologous prime-boost immunization with live attenuated *B. pertussis* followed by acellular pertussis vaccine in mice. Vaccine 2014;32:4281–8.
[89] Locht C, Papin JE, Lecher S, Debrie AS, Thalen M, Solovay K, et al. Live attenuated pertussis vaccine BPZE1 protects baboons against *Bordetella pertussis* disease and infection. J Infect Dis 2017;216:117–24.
[90] Kammoun H, Feunou PF, Foligne B, Debrie AS, Raze D, Mielcarek N, et al. Dual mechanism of protection by live attenuated *Bordetella pertussis* BPZE1 against *Bordetella bronchiseptica* in mice. Vaccine 2012;30:5864–70.
[91] Li R, Lim A, Phoon MC, Narasaraju T, Ng JK, Poh WP, et al. Attenuated *Bordetella pertussis* protects against highly pathogenic Influenza A viruses by dampening the cytokine storm. J Virol 2010;84:7105–13.
[92] Schnöller C, Roux X, Sawant D, Raze D, Olszewska W, Locht C, et al. Attenuated *Bordetella pertussis* vaccine protects against respiratory syncytial virus disease via an IL-17-dependent mechanism. Am J Respir Crit Care Med 2014;189:194–202.
[93] Netea MG, Joosten LA, Latz E, Mills KH, Natoli G, Stunnenberg HG, et al. Trained immunity: a program of innate immune memory in health and disease. Science 2016;352:aaf1098.
[94] Kavanagh H, Noone C, Cahill E, English K, Locht C, Mahon BP. Attenuated *Bordetella pertussis* vaccine strain BPZE1 modulates allergen-induced immunity and prevents allergic pulmonary pathology in a murine model. Clin Exp Allergy 2010;40:933–41.
[95] Li R, Cheng C, Chong SZ, Lim AR, Goh YF, Locht C, et al. Attenuated *Bordetella pertussis* BPZE1 protects against allergic airway inflammation and contact dermatitis in mouse models. Allergy 2012;67:1250–8.
[96] Fedele G, Bianco M, Debrie AS, Locht C, Ausiello C. Attenuated *Bordetella pertussis* vaccine candidate BPZE1 promotes human dendritic cell CCL21-induced migration and drives a Th1/Th17 response. J Immunol 2011;186:5388–96.
[97] Fedele G, Sanseverino I, D'Agostino K, Schiavoni I, Locht C, Horenstein AL, et al. Unconventional, adenosine-producing suppressor T cells induced by dendritic cells exposed to BPZE1 pertussis vaccine. J Leukoc Biol 2015;98:631–9.
[98] Skerry CM, Cassidy JP, English K, Feunou PF, Locht C, Mahon BP. A live attenuated *Bordetella pertussis* candidate vaccine does not cause disseminating infection in gamma interferon receptor knockout mice. Clin Vaccine Immunol 2009;16:1344–51.
[99] Janda WM, Santos E, Stevens J, Celig D, Terrile L, Schreckenberger PC. Unexpected isolation of *Bordetella pertussis* from a blood culture. J Clin Microbiol 1994;32:2851–3.
[100] Feunou PF, Ismaili J, Debrie AS, Huot L, Hot D, Raze D, et al. Genetic stability of the live attenuated *Bordetella pertussis* vaccine candidate BPZE1. Vaccine 2008;26:5722–7.
[101] Parkhill J, Sebaihia M, Preston A, Murphy LD, Thomson N, Harris DE, et al. Comparative analysis of the genome sequences of *Bordetella pertussis*, *Bordetella parapertussis* and *Bordetella bronchiseptica*. Nat Genet 2003;35:32–40.
[102] Thorstensson R, Trollfors B, Al-Tawil N, Jahnmatz M, Bergström J, Ljungman M, et al. A phase I clinical study of a live attenuated *Bordetella pertussis* vaccine – BPZE1; a single centre, double-blind, placebo-controlled, dose-escalating study of BPZE1 given intranasally to healthy adult male volunteers. PLoS One 2014;9:e83449.
[103] Jahnmatz M, Amu S, Ljungman M, Wehlin L, Chiodi F, Mielcarek N, et al. B-cell responses after intranasal vaccination with the novel attenuated *Bordetella pertussis* vaccine strain BPZE1 in a randomized phase I clinical trial. Vaccine 2014;32:3350–6.
[104] Wilk MM, Misiak A, McManus RM, Allen AC, Lynch MA, Mills KHG. Lung CD4 tissue-resident memory T cells mediate adaptive immunity induced by previous infection of mice with *Bordetella pertussis*. J Immunol 2017;199:233–43.